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ANTAGONISTIC ACTIVITY OF ACTINOMYCETES ISOLATED FROM SOIL SAMPLES OF SATARA DISTRICT, MAHARASTRA, INDIA AGAINST SELECTED HUMAN PATHOGENS

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ABSTRACT
The present study was undertaken to isolate, identify and determine the anti-microbial profile of antagonistic actinomycetes from different soil samples. Actinomycetes were isolated from the soil samples of two different micro-ecosystems namely, Koyna river bank and Sugar factory effluent discharge point from Satara district, Maharastra, India. 58 actinomycetes were isolated from 30 soil samples and only 12 isolates (20.69%) exhibited inhibitory activity against the sensitive *Escherichia coli* (MTCC 739). Soil samples from Koyna river bank yielded greater number of antagonistic actinomycetes (27.27%) followed by those of sugar factory effluent discharge point (12%). Nine isolates with high inhibitory activity, were further tested for their inhibitory profile against nine human test pathogens, *Staphylococcus aureus, Salmonella typhi, S. paratyphi* A, *S. typhimurium, Shigella flexneri, S. sonnei, Klebsiella pneumoniae, Vibrio parahaemolyticus* and *Candida albicans* by modified cross-streak assay. All the nine antagonistic actinomycete isolates tested (100%) exhibited antagonism at varying levels against any one of the human test pathogens. The actinomycete isolate KY-33 exhibited a high degree of inhibitory activity against all the test pathogens both in terms of the number of test pathogens inhibited and the zone of inhibition. The isolate KY-33 was subjected to standard chemotaxonomic schemes of identification. With the presence of LL-DAP, glycine, absence of characteristic sugars and G: C content of 71.98%, the isolate KY-33 was classified under cell wall chemotype-I, sugar pattern- C and was identified to be belonging to the genus *Streptomyces*. The results of present study clearly suggest that soil samples rich in organic matter with a high C:N ratio are the potential source of antagonistic actinomycetes and a large number of antagonistic actinomycetes producing a diverse classes of anti-microbial compounds can be isolated from these ecosystems.

KEY WORDS: soil, antagonistic, actinomycetes, human pathogens, TLC, hyperchromicity, *Streptomyces*

INTRODUCTION

Bioactive compounds with antimicrobial properties from various groups of organisms are being used for therapy ever since the discovery of penicillin was by Alexander Fleming in 1928. Unfortunately, in recent times, there are ever increasing reports of emergence of human pathogens with multiple drug-resistance (Spellberg *et al.,* 2004) and drug resistant human pathogens pose a huge threat in the treatment of diseases (Luzhetskyy *et al.,* 2007). This has forced the scientific community to keep on exploring for novel bio-active compounds with anti-microbial compounds. Actinomycetes which are gram-positive, filamentous bacteria are ubiquitous in soil and have been described as the greatest source of antibiotics ever since Waksman introduced streptomycetes into his systematic screening programme for the new antibiotics in the early 1940s. They have provided more than 4,000 of the naturally occurring antibiotics discovered including many of those important in medicine, such as Aminoglycosides, Anthracyclines, Chloramphenicol, β-lactams, Macrolides and Tetracyclines (Okami and Hotta, 1988) and almost 80% of the world's antibiotics are known to have their source linked to actinomycetes (Pandey *et al*., 2004). Terrestrial soil samples have also been a rich source of actinomycetes (Okazaki and Natio, 1986).Hence the present study was undertaken with the objectives of isolation of actinomycete strains from different soil samples, detection of antagonistic actinomycetes, determination of degree of antagonism towards selected human test pathogens and identification using standard chemotaxonomic schemes.

MATERIALS & METHODS

Collection of samples

5-10 grams of the soil sample was collected aseptically in sterile polypropylene bags from two different sampling stations namely, Koyna river bank and Sugar factory effluent disposal point from Satara district, Maharastra, India. A total of 30 samples, 15 soil samples from each of the two sampling stations, were collected aseptically and stored at refrigeration temperature till further use.

Isolation of actinomycetes

Soil samples were serially diluted by tenfold dilution using sterile saline. The dilutions were thoroughly mixed with a vortex mixer for a minute. Aliquots of 0.1 ml from each dilution were spread plated onto Starch-Casein Agar (SCA) (Hi-Media Pvt. Ltd., Mumbai) (Table 1), a selective medium with two anti-fungal agents, Cycloheximide and Nystatin @ 50 µg/ml (Hi-Media Pvt. Ltd. Mumbai). Plates were incubated at room temperature for 6-7 days. The actinomycete isolates were selected based on their colony morphology with a typical chalky to leathery appearance (IMTECH, 1998). They were further tested for the gram reaction, acid fast reaction and

subjected to light microscopy for filamentous nature, width of hyphae, nature of aerial and substrate mycelium (Cappucino and Sherman, 2004). The Gram-positive, non-acid fast isolates with aseptate hyphae were picked up and purified on Starch Casein Agar (SCA) plates. The purified isolates were sub-cultured on SCA slants, incubated at room temperature for 6-7 days and stored at refrigeration temperature till further use.

TABLE 1: Composition of AAM (Antibiotic Assay Medium) (g/l)

Peptic digest of Animal tissue	6.0
Yeast extract	3.0
Beef extract	1.5
Agar	15.0
D/w	Make up to 1L
pH	7.9 ± 0.2

Detection of antagonistic actinomycetes

Primary screening of the purified actinomycete isolates for their antagonistic activity, was carried out employing a modified spot inoculation method of James *et al*.(1996), against a sensitive strain of *Escherichia coli* (MTCC 739) (IMTECH, Chandigarh). The actinomycete isolates were spot inoculated at the center of Antibiotic Assay Medium (AAM) (Hi-Media Pvt. Ltd. Mumbai) (Table 2) and incubated for 6-7 days at room temperature. The plates were then flooded with an overnight broth culture of the sensitive strain of *E. coli* and incubated at 37^0C for 24-48 hours. The actinomycete strains which inhibited the growth of the sensitive *E. coli* strain as evidenced by the presence of clear zones of growth inhibition around their colony, were identified to be antagonistic. The level of inhibitory activity of the actinomycete isolates was evaluated on a 5-point scale based on the extent of the zone of inhibition. Antagonistic actinomycete isolates with higher inhibitory activity were used for the secondary screening against selected human test pathogens.

TABLE 2: Composition of SCA (Starch Casein Agar) (g/l)

Soluble starch	10.0
Vitamin free casamino acids	0.3
Calcium Carbonate $CaCO_3$	0.02
Fe3SO4.7H2O	0.01
KNO3	2.0
MgSO4.7H2O	0.05
NaCl	2.0
Agar	18.0
D/w	Make upto 1L
pH	7.1±0.1

Characterization of Inhibitory activity of antagonistic actinomycetes

Secondary screening of the antagonistic actinomycetes was carried out to understand their inhibitory profile against seven selected Gram-negative, one Gram-positive bacterial and one fungal human pathogens following the modified cross-streak assay of Lemos *et al*. (1985). Nine human test pathogens, *Salmonella typhi* (MTCC 734), *Vibrio parahaemolyticus* (MTCC 451), *Salmonella paratyphi* A (MTCC 735), *Shigella flexneri* (MTCC 1457), *Shigella sonnei* (MTCC 2957), *Salmonella typhimurium* (MTCC 98), *Klebsiella pneumoniae* (MTCC 109), *Staphylococcus aureus* (MTCC 96) and *Candida albicans* (MTCC 227), procured from IMTECH, Chandigarh were used in the assay. Inoculum from each of these inhibitory actinomycete isolates was used for making a diagonal streak on Antibiotic Assay Medium (AAM) agar plate and incubated at room temperature for a period of 6-7 days. For streaking, the inoculation loop was straightened out and bent into L-shape so as to get a streak of 8-10 mm width. After the incubation, young culture of each of the selected human test pathogens was streaked perpendicular to the central streak of the actinomycetes culture, leaving a gap of 2 mm from the central streak of the actinomycete culture. After incubation at 37^0C for 24h, the inhibitory activity of actinomycete isolates was indicated by the clear zone near the central streak, due to growth inhibition of test pathogens and this clear zone was measured in millimeters (mm) and recorded. The AAM agar plates with only the test pathogens served as control.

Identification of antagonistic actinomycetes

The actinomycetes isolates with prominent antagonistic activity were identified using the standard chemotaxonomic schemes of IMTECH (1998) and Goodfellow (1989).

Light microscopy

The isolates with prominent antagonistic activity were sub-cultured using cover slip culture technique onto SCA medium. A novel, indigenously designed cover slip holder was used for scanning the field for the nature of aerial and substrate mycelium using a phase-contrast Nikon-make microscope (Cappucino and Sherman, 2004).

Biochemical tests and determination of cell wall chemotypes

The highly inhibitory actinomycete isolates were subjected to casein, xanthine, urea, xylose and lactose utilization tests (Schaal, 1985). The extracted cell wall amino acids were subjected to Thin Layer Chromatography (TLC) using cellulose coated thin layer chromatography sheet, LL-Diamino Pimelic Acid (DPA), meso-DAP, DD-DAP isomer standards(Qualigens, India) and methanol: water: 6 N HCl: Pyridine (80: 26: 4: 10 v/v) as mobile phase. The visualization was done by spraying the plates with 0.2% (w/v) ninhydrin in acetone. The plates were heated at

105^0C for 5 minutes. Rf values of samples were calculated, compared with standards and the sugars in the samples were identified. The extracted cell wall sugars were subjected to Thin Layer Chromatography (TLC). Samples were run on silica gel coated TLC sheet with Glucose, Mannose, Rhamnose, Galactose, Ribose, Arabinose, Xylose as sugar standards (Qualigens, India), and acetonitrile :water (92.5:7.5 v/v) as mobile phase. The spots were visualized by spraying aniline phthalate reagent (prepared using aniline 2 ml, phthalic acid 3.3g and water saturated butanol 100 ml) and heating the plates at 100^0C for 5 minutes. Rf values of samples were calculated, compared with standards and the sugars in the samples were identified.

Determination of the G+C content of DNA of antagonistic actinomycete isolates

The chromosomal DNA from the antagonistic actinomycete isolates was extracted using the protocol of Wilson (2003). For hypochromicity evaluation of the chromosomal DNA of antagonistic actinomycete, the temperature was raised in increments of 2^0C from 40^0C to 96^0C and the absorbance was taken at each temperature at 260 nm using Agilent 8453 UV-Visible Spectrophotometer with thermostat controlled cell housing and a water bath. The absorbance was recorded till there was no further increase. Hypochromicity values were calculated by dividing the absorbance at each temperature by the initial absorbance at 25^0C (A_t/A_{25}). The hypochromicity curve was plotted against the different temperature points at which the absorbance was measured. Midpoint of hypochromicity curve was taken as the Tm and calculation of G+C content was done according to the formula G+C=(Tm-53.9)2.44 (Mandel & Murmur, 1968).

RESULTS

Isolation of actinomycetes

A total of 58 actinomycetes were isolated from a total of 30 soil samples. High number of actinomycete isolates was obtained from Koyna river bank followed by Sugar factory effluent discharge point (Table 3).

TABLE 3: Number of actinomycete colonies isolated from different soil samples belonging to different sampling stations

Sampling Stations	Number of Soil Samples	Number of Actinomycete Isolates
Koyna river bank	15	33
Sugar factory effluent discharge point	15	25
Total	30	58

Detection of antagonistic actinomycetes

Out of 58 actinomycete isolates from the two sampling stations, only 12 isolates inhibited the growth of the sensitive *E. coli* strain, constituting to 20.69% of the total number of isolates in the primary screening for the inhibitory actinomycetes (Table 4). Of the two sampling stations, the soil samples from Koyna river bank yielded the highest number of antagonistic actinomycetes (27.27%) followed by Sugar factory effluent discharge point (12.00%)(Table 4). The isolate KY-33 recorded the highest inhibitory score of 5 on a 5-point scale against the sensitive *E. coli* strain followed by KY-18 with a score of 4 and KY-15 & KY-31 each with a score of 3 (Fig 1).

TABLE 4: Number of antagonistic Actinomycetes isolated from various soil samples belonging to different sampling stations

Sampling Stations	Number of Actinomycete Isolates	Number of antagonistic Actinomycete Isolates
Koyna river bank	33	9 (27.27%)
Sugar factory effluent discharge point	25	3 (12.00%)
Total	58	12 (20.69%)

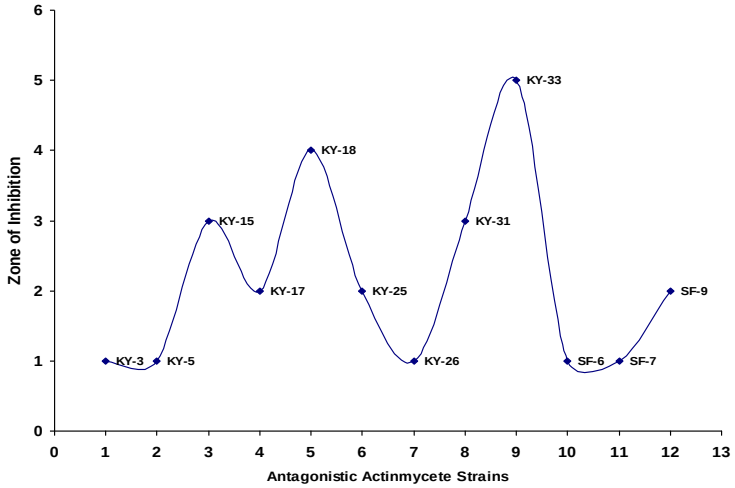


FIGURE 1: Antagonistic activity of actinomycete isolates against sensitive *E. coli* (MTCC 739) strain detected by zone of inhibition and represented on a 5-point scale. 5-Point Scale : 5- Highest level of inhibitory activity; 4- Higher inhibitory activity; 3- Moderate level of inhibitory activity; 2 -Lower level of inhibitory activity, 1- Lowest inhibitory activity; 0- No inhibitory activity

Inhibitory activity of the actinomycete isolates against various test pathogens

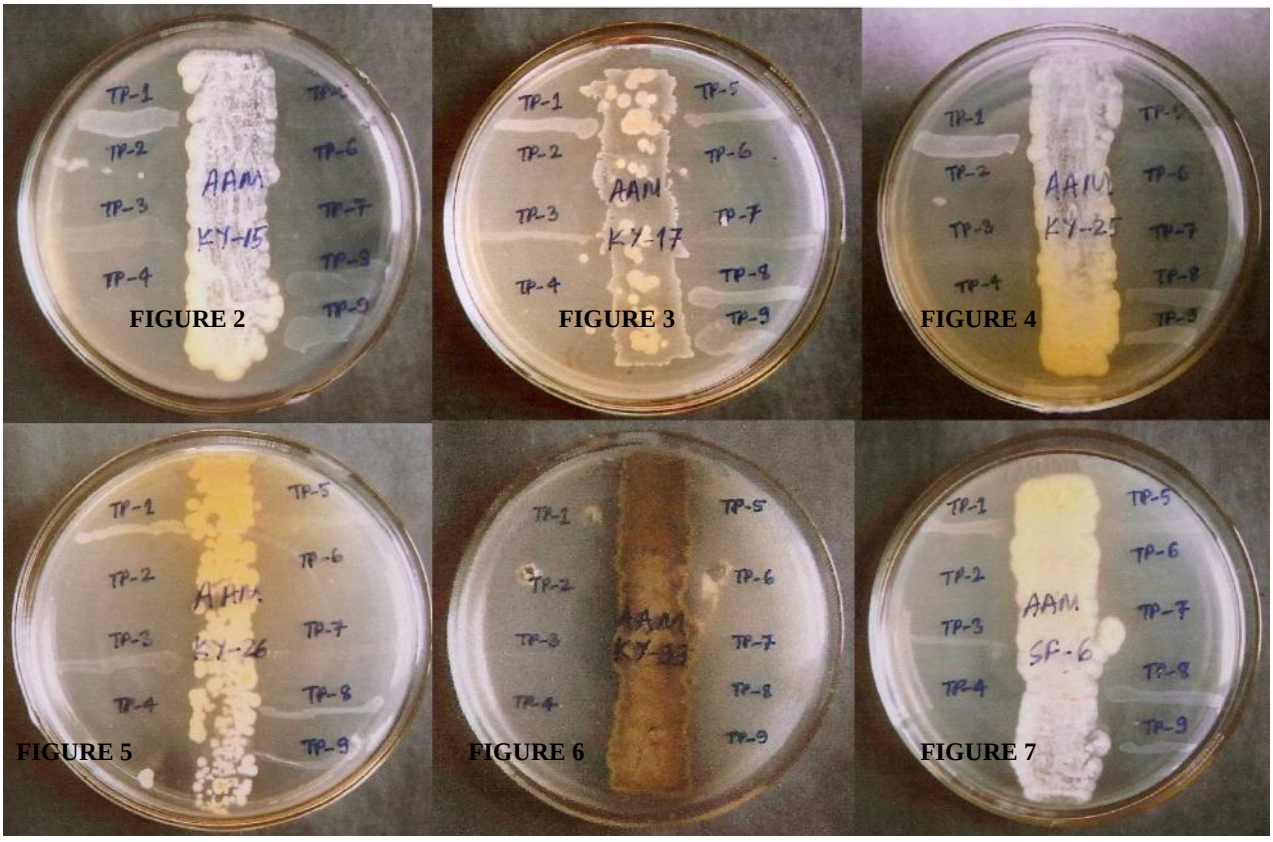
Among the 12 antagonistic isolates, nine isolates which had higher inhibitory activity were selected to determine their inhibitory spectrum against selected human test pathogens. One isolate KY-33 from the Koyna river bank exhibited prominent inhibitory activity with a zone of inhibition of ≥ 20 mm against six test pathogens and inhibited all the test pathogens. The isolates KY-15, KY-18, KY-25 and SF-7 also recorded a higher zone of inhibition of ≥ 20 mm, but against any two of the test pathogens. The isolate, KY-18 also exhibited good inhibitory activity and inhibited five of the test pathogens. The test pathogens Tp-2, *Vibrio parahaemolyticus* and Tp-6, *Shigella flexneri* did not show any growth on AAM medium and hence were not considered in the assay results(Table 5) (Fig. 2 -7).

TABLE 5: Zone of inhibition of actinomycete isolates by cross-streak assay against various test pathogens

Actinomycete	Inhibition of Test Pathogens (zone expressed in mm)								
Isolate	Tp-1	Tp-2	Tp-3	Tp-4	Tp-5	Tp-6	Tp-7	Tp-8	Tp-9
KY-3	R	NGM	R	12	R	NGM	27	R	R
KY-15	R	NGM	R	23	7	NGM	27	R	R
KY-17	4	NGM	R	26	R	NGM	11	R	R
KY-18	R	NGM	18	22	14	NGM	25	8	R
KY-25	R	NGM	R	24	11	NGM	24	R	R
KY-31	R	NGM	R	21	R	NGM	18	R	R
KY-33	30	NGM	18	31	30	NGM	31	29	30
SF-6	R	NGM	R	19	R	NGM	24	R	R
SF-9	R	NGM	29	18	R	NGM	R	24	R

Tp – Test Pathogen, NGM- No Growth on the Medium AAM, R- Resistant

Test Pathgoens: Tp-1, *Staphylococcus aureus*; Tp-2, *Vibrio parahaemolyticus* ; Tp-3, *Salmonella typhi*; Tp-4, *Candida albicans*; Tp-5, *Salmonella paratyphi A*; Tp-6, *Shigella flexneri*; Tp-7, *Shigella sonnei*; Tp-8, *Salmonella typhimurium*; Tp-9, *Klebsiella pneumonia*



FIGURES 2 -7: Inhibitory activity profile of antagonistic actinomycetes against human test pathogens by cross-streak assay, Test Pathgoens: Tp-1, *Staphylococcus aureus*; Tp-2, *Vibrio parahaemolyticus* ; Tp-3, *Salmonella typhi*; Tp-4, *Candida albicans*; Tp-5, *Salmonella paratyphi A*; Tp-6, *Shigella flexneri*; Tp-7, *Shigella sonnei*; Tp-8, *Salmonella typhimurium*; Tp-9, *Klebsiella pneumoniae*

Identification of actinomycete isolates with antagonistic activity

All the 12 isolates with inhibitory activity against the sensitive strain of *E. coli* (MTCC 739) were gram positive and non-acid fast (Table 6). The isolate, KY-33 which showed prominent inhibitory activity against all the test pathogens, was subjected to chemotaxonomic scheme of identification. During the TLC analysis of cell wall hydrolysate for the amino acids, LL-DAP and glycine were present in the isolate, KY-33 and hence it was classified under Cell wall chemotype-I. The whole cell sugar analysis of the isolate KY-33 using TLC, showed absence of characteristic sugars (Table 6) and hence was classified under the sugar pattern- C. The temperature of melting (Tm) as calculated from the hypochromicity curve was 83.4^0C (Fig 8) and the G:C content of the genomic DNA was estimated to be 71.98% (Table 6). From the above results, the isolate KY-33 was identified to be belonging to the genus *Streptomyces* (Table 6).

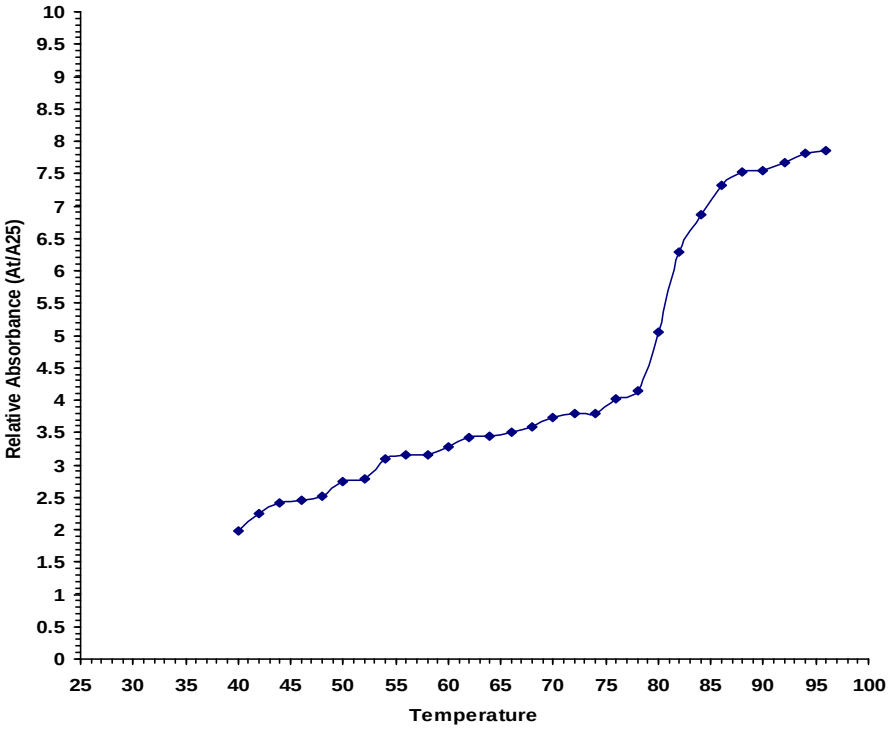


FIGURE 8: Hyperchromicity curve of chromosomal DNA of the antagonistic actinomycete isolate KY-33

TABLE 6: Tests used for the identification of the actinomycete isolate KY-33

Test/Analysis		Result
	Gram Reaction	Gram +ve
	Acid-Fast Staining	Non acid-fast
Light Microscopy	Cellular Nature	Filamentous, Asepatate hyphae with hyphal width -0.5 - 2 µ Aerial hyphae- bear spores in spirals
	Casein decomposition	+
	Xanthine decomposition	+
Biochemical Tests	Urea decomposition	+
	Acid from Xylose	+
	Acid from Lactose	+
Cell wall amino acid	LL-DAP	Present Cell wall chemotype-I
TLC Analysis	Meso-DAP	Absent
	DD-DAP	Absent
Whole cell sugar pattern		Sugar pattern - C No diagnostic sugar present
G:C content of genomic DNA		71.98%

DISCUSSION

Isolation of actinomycetes

In the present study, high number of actinomycete isolates was obtained from Koyna river bank followed by Sugar factory effluent discharge point (Table 3). Goodfellow and Simpson (1987) reported that actinomycetes are found abundantly in cultivated and uncultivated soils in various regions throughout the world. Actinomycetes were

isolated by many workers from various soil samples including river bank soil (Okudoh and Wallis, 2007; Velayudham and Murugan, 2012; Algafari, 2014; Bakheit and Saadabi, 2014; Patel *et al*., 2014).

Detection and Determination of antagonistic activity of actinomycetes

In the present study, samples from Koyna river bank yielded a high percentage (27.27%) of antagonistic actinomycetes when compared to that of sugar factory effluent discharge point (12.00%) antagonistic to the sensitive *E. coli* strain (Table 4) (Fig 1). Contradictory to the present findings, Okudoh and Wallis (2007), in their study, reported 94 actinomycete isolates from the riverine soil samples but found none of them inhibitory. However, they also reported that 3.11% of the actinomycetes isolated from different soil samples were antagonistic. In another study, Velayudham and Murugan (2012) observed that 97.22% of the actinomycete isolates were antagonistic. In the present study, all the nine (100%) of the antagonistic actinomycete isolates were inhibitory to the test pathogen, *Candida albicans* followed by eight (88.89%) isolates against *Shigella sonnei*, 4 (44.44%) against *Salmonella paratyphi* A, 3 (33.33%) against *Salmonella typhi*, 3 (33.33%) against *Salmonella typhimurium*, 2 (22.22%) against *Staphylococcus aureus*, 1 (11.11%) against *Klebsiella pneumoniae* (Table 5)(Fig. 2-7). However, Velayudham and Murugan (2012) in their study, reported that 55.56% of the isolates were inhibitory to *Candida albicans* and 47.22% of the isolates were inhibitory to *Staphylococcus aureus*. However, Patel *et al*.(2014), reported that 44.03% isolates were antagonistic to *Staphylococcus aureus* and 9.7% showed activity against *Candida albicans.* However, in the present study, all the actinomycete isolates tested (100%) exhibited antagonism at varying levels against any one of the test pathogens (Table 5). In a study Patel *et al.*(2014) observed that 79% of the actinomycete isolates had inhibitory activity against one or more pathogens.

In the present study, the soil samples from the Koyna river bank yielded highest number of antagonistic actinomycetes (27.27%) when compared to sugar factory effluent discharge point (12.00%) (Table 4). The reasons for high incidence of actinomycetes in general and antagonistic actinomycetes in specific, in the soil samples from Koyna river bank may be due to the fact that the soil samples were collected from an area contaminated with sewage from a sewage discharge trench. Because of high organic load and high Carbon to Nitrogen (C:N) ratio, these soil samples favor the growth of actinomycetes as well as other bacteria also, leading to high competition between actinomycetes and other bacterial species for nutrients & space. In such micro-environments with high bacterial load, only the antagonistic actinomycetes thrive in high numbers both in terms of biodiversity as well as biomass by secreting highly diverse classes of anti-microbial compounds and inhibiting the growth of other microbes. The relationship between the high incidence of antagonistic actinomycetes and the polluted environments has been established by Walker and Colwell (1975), who reported that greater numbers of actinomycetes were associated with polluted environment.

On the contrary, even though the sugar factory effluent discharge point was rich in organic matter, the number of actinomycetes and antagonistic actinomycetes isolated from this sampling station were comparatively lower in the present study. This may be due to the fact that, the soil samples contaminated with sugar factory effluents contain very high levels of TDS (2,560-3,978ppm), BOD (2,225-4,526 ppm) and COD (10,896-16,843 ppm) making the environment unfavourable for the growth of microbial flora (Kaur *et al*., 2010).

Identification of antagonistic actinomycetes

In the present study, the antagonistic actinomycete isolate KY-33 with prominent inhibitory activity against all the test pathogens, was subjected to chemotaxonomic schemes of identification and was identified to be belonging to the genus *Streptomyces* spp. (Table 6). Rakshanya *et al.* (2011) reported that 60% of the antagonistic actinomycetes isolates were identified to be belonging to *Streptomyces sp.* In another study, Bakheit and Saadabi (2014) observed that 76% of their actinomycete isolates were belonging to the genus *Streptomyces.* However, 100% of the isolated antagonistic actinomycetes were identified to be belonging to *Streptomyces sp*. in a study by Elamvazhuthi and Subramanian (2013). Studies have shown that the soils rich in organic matter with high carbon support higher biomass of *Strepomyces spp.* (Lee and Hwang, 2002; Bonjar, 2004).

The results of the present study clearly suggest that soil samples are the great sources of actinomycetes and that actinomycetes are the greatest source of diverse anti-microbial compounds. In particular, large number of antagonistic actinomycetes can be isolated from the soil samples of ecosystems, which are rich in organic matter with a high Carbon to Nitrogen ratio.

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